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(54) Novel bacillus thuringiensis isolate active against lepidopteran pests, and genes encoding novel lepidopteran-active toxins

Isolat aus Bacillus thuringiensis, das gegen Lepidoptera-Schädlinge aktiv ist, und Gene, die für die neuen lepidoptera-aktiven Toxine kodieren

Isolat de Bacillus thuringiensis actif contre les animaux nuisibles de lépidoptera, et gènes encodant les toxines actives à lepidoptera

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Description

Background of the Invention

The most widely used microbial pesticides are derived from the bacterium Bacillus thuringiensis. This bacterial agent is used to control a wide range of leaf-eating caterpillars and beetles, as well as mosquitos. Bacillus thuringiensis produces a proteinaceous parasporal body or crystal which is toxic upon ingestion by a susceptible insect host. For example, B. thuringiensis subsp. kurstaki HD-1 produces a crystal inclusion consisting of a biotoxin called a delta toxin which is toxic to the larvae of a number of lepidopteran insects. The cloning, sequencing, and expression of this B.t. crystal protein gene in Escherichia coli has been described in the published literature (Schnepf, H.E. and Whitely, H.R. [1981] Proc. Natl. Acad. Sci. USA 78:2893-2897; Schnepf et al.). U.S. Patent 4,448,885 and U.S. Patent 4,467,036 both disclose the expression of B.t. crystal protein in E. coli.

Brief Summary of the Invention

The subject invention concerns a novel Bacillus thuringiensis isolate designated B.t. PS81I which has activity against all lepidopteran pests tested.

Also disclosed and claimed are novel toxin genes which express toxins toxic to lepidopteran insects. These toxin genes can be transferred to suitable hosts via a plasmid vector.

Specifically, the invention comprises the novel B.t. isolate denoted B.t. PS81I, mutants thereof, and novel δ-endotoxin genes derived from this B.t. isolate which encode proteins which are active against lepidopteran pests.

Detailed Disclosure of the Invention

The novel toxin genes of the subject invention were obtained from a novel lepidopteran-active B. thuringiensis (B.t.) isolate designated PS81I.

Characteristics of *B.t.* PS81I

Colony morphology -- Large colony, dull surface, typical B.t.
Vegetative cell morphology -- typical B.t.
Flagellar serotype -- 7, aizawai.
Intracellular inclusions -- sporulating cells produce a bipyramidal crystal.
Plasmid preparations--agarose gel electrophoresis of plasmid preparations distinguishing B.t. PS81I from B.t. HD-1.
Alkali-soluble proteins -- SDS-PAGE analysis shows a protein band at ca. 130,000 daltons.
Unique toxins -- four unique toxins have been identified in B.t. PS81I.
Activity -- B.t. PS81I kills all Lepidoptera tested.
Bioassay procedures:
B.t. PS81I spores and crystals were tested against: Beet Armyworm, Spodoptera exigua; Diamondback Moth, Plutella xylostella; Western Spruce Budworm, Choristoneura occidentalis.
LC50 values were as follows:

Beet Armyworm - 2.53 ppm
Diamondback Moth - 0.16 ppm
Western Spruce Budworm - 3.2 ppm

Bioassay procedure: dilutions are prepared of a spore and crystal pellet, mixed with USDA Insect Diet (Technical Bulletin 1528, U.S. Department of Agriculture), and poured into small plastic trays. Larvae are placed on the diet mixture and held at 25°C (late 2nd instar Diamondback Moth larvae, early 2nd instar Beet Armyworm larvae, 4th instar Western Spruce Budworm larvae). Mortality is recorded after six days.

B. thuringiensis PS81I, NRRL B-18484, and mutants thereof, can be cultured using standard known media and fermentation techniques. Upon completion of the fermentation cycle, the bacteria can be harvested by first separating the B.t. spores and crystals from the fermentation broth by means well known in the art. The recovered B.t. spores and crystals can be formulated into a wettable powder, a liquid concentrate, granules or other formulations by the addition of surfactants, dispersants, inert carriers and other components to facilitate handling and application for particular target pests. The formulation and application procedures are all well known in the art and are used with commercial strains of B. thuringiensis (HD-1) active against Lepidoptera, e.g., caterpillars. B.t. PS81I, and mutants thereof, can be used to control lepidopteran pests.

A subculture of B.t. PS81I and the E. coli hosts harboring the toxin genes of the invention, were deposited in the permanent collection of the Northern Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois, USA. The accession numbers and deposit dates are as follows:

Subculture	Accession Number	Deposit Date
B.t. PS81I	NRRL B-18484	April 19, 1989
E. coli(NM522)(pMYC392)	NRRL B-18498	May 17, 1989
E. coli (NM522)(pMYC393)	NRRL B-18499	May 17, 1989
E. coli (NM522)(pMYC394)	NRRL B-18500	May 17, 1989
E. coli (NM522)(pMYC1603)	NRRL B-18517	June 30, 1989

The toxin genes of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., Pseudomonas, the microbes can be applied to the situs of lepidopteran insects where they will proliferate and be ingested by the insects. The result is a control of the unwanted insects. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the B.t. toxin.

Where the B.t. toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera Bacillus, Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, and Alcaligenes; fungi, particularly yeast, e.g., genera Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula, and Aureobasidium. Of particular interest are such phytosphere bacterial species as Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus, and Azotobacter vinlandii; and phytosphere yeast species such as Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus,C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae, and Aureobasidium pollulans. Of particular interest are the pigmented microorganisms.

A wide variety of ways are available for introducing a B.t. gene expressing a toxin into the microorganism host under conditions which allow for stable maintenance and expression of the gene. One can provide for DNA constructs which include the transcriptional and translational regulatory signals for expression of the toxin gene, the toxin gene under their regulatory control and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional in the host, whereby integration or stable maintenance will occur.

The transcriptional initiation signals will include a promoter and a transcriptional initiation start site. In some instances, it may be desirable to provide for regulative expression of the toxin, where expression of the toxin will only occur after release into the environment. This can be achieved with operators or a region binding to an activator or enhancers, which are capable of induction upon a change in the physical or chemical environment of the microorganisms. For example, a temperature sensitive regulatory region may be employed, where the organisms may be grown up in the laboratory without expression of a toxin, but upon release into the environment, expression would begin. Other techniques may employ a specific nutrient medium in the laboratory, which inhibits the expression of the toxin, where the nutrient medium in the environment would allow for expression of the toxin. For translational initiation, a ribosomal binding site and an initiation codon will be present.

Various manipulations may be employed for enhancing the expression of the messenger RNA, particularly by using an active promoter, as well as by employing sequences, which enhance the stability of the messenger RNA. The transcriptional and translational termination region will involve stop codon(s), a terminator region, and optionally, a polyadenylation signal. A hydrophobic "leader" sequence may be employed at the amino terminus of the translated polypeptide sequence in order to promote secretion of the protein across the inner membrane.

In the direction of transcription, namely in the 5′ to 3′ direction of the coding or sense sequence, the construct will

involve the transcriptional regulatory region, if any, and the promoter, where the regulatory region may be either 5′ or 3′ of the promoter, the ribosomal binding site, the initiation codon, the structural gene having an open reading frame in phase with the initiation codon, the stop codon(s), the polyadenylation signal sequence, if any, and the terminator region. This sequence as a double strand may be used by itself for transformation of a microorganism host, but will usually be included with a DNA sequence involving a marker, where the second DNA sequence may be joined to the toxin expression construct during introduction of the DNA into the host.

By a marker is intended a structural gene which provides for selection of those hosts which have been modified or transformed. The marker will normally provide for selective advantage, for example, providing for biocide resistance, e.g., resistance to antibiotics or heavy metals; complementation, so as to provide prototropy to an auxotrophic host, or the like. Preferably, complementation is employed, so that the modified host may not only be selected, but may also be competitive in the field. One or more markers may be employed in the development of the constructs, as well as for modifying the host. The organisms may be further modified by providing for a competitive advantage against other wild-type microorganisms in the field. For example, genes expressing metal chelating agents, e.g., siderophores, may be introduced into the host along with the structural gene expressing the toxin. In this manner, the enhanced expression of a siderophore may provide for a competitive advantage for the toxin-producing host, so that it may effectively compete with the wild-type microorganisms and stably occupy a niche in the environment.

Where no functional replication system is present, the construct will also include a sequence of at least 50 basepairs (bp), preferably at least about 100 bp, and usually not more than about 1000 bp of a sequence homologous with a sequence in the host. In this way, the probability of legitimate recombination is enhanced, so that the gene will be integrated into the host and stably maintained by the host. Desirably, the toxin gene will be in close proximity to the gene providing for complementation as well as the gene providing for the competitive advantage. Therefore, in the event that a toxin gene is lost, the resulting organism will be likely to also lose the complementing gene and/or the gene providing for the competitive advantage, so that it will be unable to compete in the environment with the gene retaining the intact construct.

A large number of transcriptional regulatory regions are available from a wide variety of microorganism hosts, such as bacteria, bacteriophage, cyanobacteria, algae, fungi, and the like. Various transcriptional regulatory regions include the regions associated with the trp gene, lac gene, gal gene, the lambda left and right promoters, the Tac promoter, the naturally-occurring promoters associated with the toxin gene, where functional in the host. See for example, U.S. Patent Nos. 4,332,898, 4,342,832 and 4,356,270. The termination region may be the termination region normally associated with the transcriptional initiation region or a different transcriptional initiation region, so long as the two regions are compatible and functional in the host.

Where stable episomal maintenance or integration is desired, a plasmid will be employed which has a replication system which is functional in the host. The replication system may be derived from the chromosome, an episomal element normally present in the host or a different host, or a replication system from a virus which is stable in the host. A large number of plasmids are available, such as pBR322, pACYC184, RSF1010, pRO1614, and the like. See for example, Olson et al., (1982) J. Bacteriol. 150:6069, and Bagdasarian et al., (1981) Gene 16:237, and U.S. Patent Nos. 4,356,270, 4,362,817, and 4,371,625.

The B.t. gene can be introduced between the transcriptional and translational initiation region and the transcriptional and translational termination region, so as to be under the regulatory control of the initiation region. This construct will be included in a plasmid, which will include at least one replication system, but may include more than one, where one replication system is employed for cloning during the development of the plasmid and the second replication system is necessary for functioning in the ultimate host. In addition, one or more markers may be present, which have been described previously. Where integration is desired, the plasmid will desirably include a sequence homologous with the host genome.

The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for pesticidal activity.

Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and -positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiceae, such as Rhizobium; Spirillaceae, such as photobacterium, Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillaceae; Pseudomonadaceae, such as Pseudomonas and Acetobacter; Azotobacteraceae, Actinomycetales, and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast,

such as Rhodotorula, Aureobasidium, Sporobolomyces, and the like.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the B.t. gene into the host, availability of expression systems, efficiency of expression, stability of the pesticide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp.; phylloplane organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms as Escherichia, Lactobacillus sp., Bacillus sp., Streptomyces sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis, Streptomyces lividans and the like.

The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

Treatment of the microbial cell, e.g., a microbe containing the B.t. toxin gene, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability in protecting the toxin. Examples of chemical reagents are halogenating agents, particularly halogens of atomic no. 17-80. More particularly, iodine can be used under mild conditions and for sufficient time to achieve the desired results. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Lugol iodine, Bouin's fixative, and Helly's fixative (See: Humason, Gretchen L., Animal Tissue Techniques, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host animal. Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like.

The cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of inactivation should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

The cellular host containing the B.t. insecticidal gene may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the B.t. gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

The B.t. cells may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

The pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the pesticide while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

The formulations can be applied to the environment of the lepidopteran pest(s), e.g., plants, soil or water, by spraying, dusting, sprinkling, or the like.

Mutants of PS81I can be made by procedures well known in the art. For example, an asporogenous mutant can be obtained through ethylmethane sulfonate (EMS) mutagenesis of PS81I. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A smaller percentage of the asporogenous mutants will remain intact and not lyse for extended fermentation periods; these strains are designated lysis minus (-). Lysis minus strains can be identified by screening asporogenous mutants in shake flask media and selecting those mutants that are still intact and contain toxin crystals at the end of the fermentation. Lysis minus strains are suitable for a cell fixation process that will yield a protected, encapsulated toxin protein.

To prepare a phage resistant variant of said asporogenous mutant, an aliquot of the phage lysate is spread onto nutrient agar and allowed to dry. An aliquot of the phage sensitive bacterial strain is then plated directly over the dried

lysate and allowed to dry. The plates are incubated at 30°C. The plates are incubated for 2 days and, at that time, numerous colonies could be seen growing on the agar. Some of these colonies are picked and subcultured onto nutrient agar plates. These apparent resistant cultures are tested for resistance by cross streaking with the phage lysate. A line of the phage lysate is streaked on the plate and allowed to dry. The presumptive resistant cultures are then streaked across the phage line. Resistant bacterial cultures show no lysis anywhere in the streak across the phage line after overnight incubation at 30°C. The resistance to phage is then reconfirmed by plating a lawn of the resistant culture onto a nutrient agar plate. The sensitive strain is also plated in the same manner to serve as the positive control. After drying, a drop of the phage lysate is plated in the center of the plate and allowed to dry. Resistant cultures showed no lysis in the area where the phage lysate has been placed after incubation at 30°C for 24 hours.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 - Culturing *B.t.* PS81I

A subculture of B.t. PS81I, or mutants thereof, can be used to inoculate the following medium, a peptone, glucose, salts medium.

Bacto Peptone	7.5 g/l
Glucose	1.0 g/l
KH_2PO_4	3.4 g/l
K_2HPO_4	4.35 g/l
Salt Solution	5.0 ml/l
$CaCl_2$ Solution	5.0 ml/l

Salts Solution (100 ml)

$MgSO_4.7H_2O$	2.46 g
$MnSO_4.H_2O$	0.04 g
$ZnSO_4.7H_2O$	0.28 g
$FeSO_4.7H_2O$	0.40 g

$CaCl_2$ Solution (100 ml)

$CaCl_2.2H_2O$	3.66 g
pH	7.2

The salts solution and $CaCl_2$ solution are filter-sterilized and added to the autoclaved and cooked broth at the time of inoculation. Flasks are incubated at 30°C on a rotary shaker at 200 rpm for 64 hr.

The above procedure can be readily scaled up to large fermentors by procedures well known in the art.

The B.t. spores and/or crystals, obtained in the above fermentation, can be isolated by procedures well known in the art. A frequently-used procedure is to subject the harvested fermentation broth to separation techniques, e.g., centrifugation.

Example 2 - Cloning of Novel Toxin Genes From Isolate PS81I and Transformation into *Escherichia coli*

Total cellular DNA was prepared from B.t. cells grown to a low optical density (OD_{600} = 1.0). The cells were recovered by centrifugation and protoplasted in TES buffer (30 mM Tris-Cl, 10 mM ethylenediaminetetraacetic acid [EDTA], 50 mM NaCl, pH = 8.0) containing 20% sucrose and 50 mg/ml lysozyme. The protoplasts were lysed by addition of sodium dodecyl sulfate (SDS) to a final concentration of 4%. The cellular material was precipitated overnight at 4°C in 100 mM (final concentration) neutral potassium chloride. The supernate was extracted twice with phenol/chloroform (1:1). The

DNA was precipitated with ethanol and purified by isopycnic banding on a cesium gradient.

Total cellular DNA from PS81I and B.t.k. HD-1 was digested with EcoRI and separated by electrophoresis on a 0.8% (w/v) Agarose-TAE (50 mM Tris-Cl, 20 mM NaOAc, 2.5 mM EDTA, pH=8.0) buffered gel. A Southern blot of the gel was hybridized with a [^{32}P] radiolabeled probe against the 3.2 Kb NsiI to NsiI fragment of the toxin gene contained in plasmid pM3,130-7 of NRRL B-18332 and the 2.4 Kb NsiI to KpnI fragment of the "4.5 Kb class" toxin gene (Kronstad and Whitely [1986] Gene USA 43:29-40). These two fragments were combined and used as the probe. Results show that hybridizing fragments of PS81I are distinct from those of HD-1. Specifically, in the 1.5 Kb to 2.5 Kb size range, 2.3 Kb, 1.95 Kb, and 1.6 Kb hybridizing bands were detected in PS81I instead of the single 1.9 Kb hybridizing band in HD-1.

The following description outlines the steps taken in cloning two of the three EcoRI fragments described above. Two hundred micrograms of PS81I total cellular DNA was digested with EcoRI and separated by electrophoresis on a preparative 0.8% (w/v) Agarose-TAE gel. The 1.5 Kb to 2.3 Kb region of the gel was cut out and the DNA from it was electroeluted and concentrated using an ELUTIP™-d (Schleicher and Schuell, Keene, NH) ion exchange column according to the manufacturer's specification. The isolated EcoRI fragments were ligated to LAMBDA ZAP™ EcoRI arms (Stratagene Cloning Systems, La Jolla, CA) and packaged using Gigapak GOLD™ (Stratagene) extracts. The packaged recombinant phage were plated with E. coli strain BB4 (Stratagene) to give high plaque density. The plaques were screened by standard nucleic acid hybridization procedures with radiolabeled probe. The plaques that hybridized were purified and re-screened at a lower plaque density. The resulting purified phage were grown with R408 M13 helper phage (Stratagene) and the recombinant BlueScript™ (Stratagene) plasmid was automatically excised and packaged. The "phagemid" was re-infected in XL1-Blue E. coli cells (Stratagene) as part of the automatic excision process. The infected XL1-Blue cells were screened for ampicillin resistance and the resulting colonies were analyzed by a standard rapid plasmid purification procedure to identify the desired plasmids. The plasmids, designated pM2,31-4 and pM2,31-1, contain approximately 1.95 Kb and 1.6 Kb EcoRI inserts, respectively. The DNA sequence of both inserts was determined using Stratagene's T7 and T3 oligonucleotide primers plus a set of existing internal B.t. endotoxin gene oligonucleotide primers. About 500 bp of the insert in pM2,31-4 was sequenced. In the same manner, approximately 1.0 Kb of the insert in pM2,31-1 was sequenced. Data analysis comparing the two sequences to other cloned and sequenced B.t. endotoxin genes showed that two distinct, unique partial toxin gene sequences had been found. Synthetic oligonucleotides were constructed to regions in both sequences that had minimum homology to other characterized B.t. endotoxin genes. The 42-mer oligonucleotide constructed to the sequence of the insert in pM2,31-4 was GGATACCGGTGACCCATTAACATTCCAATCTTTTAGTTACGC; it was used to isolate a toxin gene sequence called 81IA. The 40-mer oligonucleotide constructed to the sequence of the insert in pM2,31-1 was GAAGTTTATGGCCTCTTTCTGTAGAAAATCAAATTGGACC; it was used to isolate a toxin gene sequence called 81IB.

In order to clone both complete toxin genes, a Sau3A partial library was constructed. PS81I total cellular DNA partially digested with Sau3A and size fractionated by electrophoresis into a mixture of 9-23 Kb fragments on a 0.6% agarose-TAE gel, and purified as described previously, was ligated into LambdaGEM-11™ (PROMEGA). The packaged phage were plated on P2392 E. coli cells (Stratagene) at a high titer and screened using the radiolabeled synthetic oligonucleotides (aforementioned) as nucleic acid hybridization probes. Hybridizing plaques, using each probe, were rescreened at a lower plaque density. Purified plaques that hybridized with either probe were used to infect P2392 E. coli cells in liquid culture for preparation of phage for DNA isolation. DNA was isolated by standard procedures. Preparative amounts of DNA were digested with SalI (to release the inserted DNA from lambda arms) and separated by electrophoresis on a 0.6% agarose-TAE gel. The large fragments, electroeluted and concentrated as described above, were ligated to SalI-digested and dephosphorylated pUC19 (NEB). The ligation mix was introduced by transformation into DH5(α) competent E. coli cells (BRL) and plated on LB agar containing ampicillin, isopropyl-(β)-D-thiogalactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-(β)-D-galactoside (XGAL). White colonies, with prospective insertions in the (β)-galactosidase gene of pUC19, were subjected to standard rapid plasmid purification procedures to isolate the desired plasmids. Plasmid pM3,122-1 contains a 15 Kb Sau3A fragment isolated using the 81IA oligonucleotide probe. Plasmid pM4,59-1 contains an 18 Kb Sau3A fragment isolated using the 81IB oligonucleotide probe.

Plasmid pM3,122-1 was digested with several restriction enzymes and Southern blotted. The blot was probed with the [^{32}P] radiolabeled 81IA specific oligonucleotide probe, as well as the labeled oligonucleotide sequencing primers made to known B.t.k. toxin genes. The resulting autoradiogram showed that two toxin genes were present in tandem on this cloned Sau3A fragment. Plasmid pM3,122-1 had a 4.0 Kb NdeI fragment that hybridized with oligonucleotide probes made to known B.t.k. genes. This fragment, however, did not hybridize with the specific oligonucleotides to 81IA or 81IB; a new toxin gene had been discovered and subsequently was called 81IA2. The 4.0 Kb NdeI fragment was isolated and cloned in pUC19, yielding plasmid pMYC392. The 81IA toxin gene was isolated by digesting pM3,122-1 with HindIII, with resulting deletion of most of the 81IA2 toxin gene. The fragment was recircularized to form pMYC1603. The 81IA toxin gene is unique based on its restriction map and is presently being sequenced.

Plasmid pM4,59-1 was digested with several restriction enzymes and Southern blotted. The blot was probed with the [^{32}P] radiolabeled 81IB specific oligonucleotide probe, as well as with labeled oligonucleotide sequencing primers made to known B.t.k. toxin genes. The plasmid pM4,59-1 was mapped and found to contain only a partial 81IB toxin

gene. The full open reading frame (ORF) of a second toxin gene was discovered on the 18 Kb fragment and called 81IB2. The 81IB2 toxin gene was cloned separately from the 81IB toxin gene by digestion of pM4,59-1 with NdeI and SmaI, filling in the NdeI overhang and ligating the linear fragment back together. The resulting plasmid was called pMYC394. The full ORF of the 81IB toxin gene was isolated from another Sau3A fragment, cloned from the lambda library, on a 7.3 Kb HindIII fragment in pBluescript (Stratagene). The resulting plasmid is pMYC393.

The toxin genes were sequenced by the standard Sanger dideoxy chain termination method using oligonucleotide primers made to the "4.5 Kb class" toxin gene and by "walking" with primers made to the sequences of the new toxin genes. Sequence analysis of the four toxin genes has elucidated unique open reading frames and has deduced unique endotoxin proteins. The toxin of this invention is shown in Chart A. The ORF is 3716 bp and the deduced endotoxin molecular weight is 133,621 daltons.

Endotoxin proteins have been expressed in Pseudomonas and/or Bacillus from the toxin genes. SDS-PAGE/Western blot analysis, using polyclonal antibodies directed against the "6.6 Kb" class toxin, verified that each gene encodes an immunoreactive protein of approximately 130,000 daltons. The toxin proteins encoded by the genes of the subject invention expressed in either a Bacillus or Pseudomonas host have activity against all lepidopteran insects tested: Trichoplusia ni, Spodoptera exigua, Plutella xylostella, and Choristoneura occidentalis.

The above cloning procedures were conducted using standard procedures unless otherwise noted.

The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. Also, methods for the use of lambda bacteriophage as a cloning vehicle, i.e., the preparation of lambda DNA, in vitro packaging, and transfection of recombinant DNA, are well known in the art. These procedures are all described in Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. Thus, it is within the skill of those in the genetic engineering art to extract DNA from microbial cells, perform restriction enzyme digestions, electrophorese DNA fragments, tail and anneal plasmid and insert DNA, ligate DNA, transform cells, prepare plasmid DNA, electrophorese proteins, and sequence DNA.

The restriction enzymes disclosed herein can be purchased from Bethesda Research Laboratories, Gaithersburg, MD, New England Biolabs, Beverly, MA, or Boehringer-Mannheim, Indianapolis, IN. The enzymes are used according to the instructions provided by the supplier.

The plasmids containing the B.t. toxin genes can be removed from the transformed host microbes by use of standard well-known procedures. For example, the host microbes can be subjected to cleared lysate isopycnic density gradient procedures, and the like, to recover the desired plasmid.

Example 3 - Insertion of Toxin Genes Into Plants

The novel genes coding for the novel insecticidal toxins, as disclosed herein, can be inserted into plant cells using the Ti plasmid from Agrobacter tumefaciens. Plant cells can then be caused to regenerate into plants (Zambryski, P., Joos, H., Gentello, C., Leemans, J., Van Montague, M. and Schell, J [1983] Cell 32:1033-1043). A particularly useful vector in this regard is pEND4K (Klee, H.J., Yanofsky, M.F. and Nester, E.W. [1985] Bio/Technology 3:637-642). This plasmid can replicate both in plant cells and in bacteria and has multiple cloning sites for passenger genes. The toxin gene, for example, can be inserted into the BamHI site of pEND4K, propagated in E. coli, and transformed into appropriate plant cells.

Example 4 - Cloning of Novel *B. thuringiensis* Genes Into Baculoviruses

The novel genes of the invention can be cloned into baculoviruses such as Autographa californica nuclear polyhedrosis virus (AcNPV). Plasmids can be constructed that contain the AcNPV genome cloned into a commercial cloning vector such as pUC8. The AcNPV genome is modified so that the coding region of the polyhedrin gene is removed and a unique cloning site for a passenger gene is placed directly behind the polyhedrin promoter. Examples of such vectors are pGP-B6874, described by Pennock et al. (Pennock, G.D., Shoemaker, C. and Miller, L.K. [1984] Mol. Cell. Biol. 4:399-406), and pAC380, described by Smith et al. (Smith, G.E., Summers, M.D. and Fraser, M.J. [1983] Mol Cell. Biol. 3:2156-2165). The gene coding for the novel protein toxin of the invention can be modified with BamHI linkers at appropriate regions both upstream and downstream from the coding region and inserted into the passenger site of one of the AcNPV vectors.

The nucleotide sequence encoding the novel B.t. toxin, and the deduced amino-acid sequence, is shown in Chart A.

It is well known in the art that the amino-acid sequence of a protein is determined by the nucleotide sequence of the DNA. Because of the redundancy of the genetic code, i.e. more than one coding nucleotide triplet (codon) can be used for most of the amino-acids used to make proteins, different nucleotide sequences can code for a particular amino-acid.

The novel B.t. toxin can be prepared via any nucleotide sequence (equivalent to that shown) encoding the same amino-acid sequence; the present invention includes such equivalent nucleotide sequences.

It has been shown that proteins of identified structure and function may be constructed by changing the amino-acid sequence, if such changes do not alter the protein secondary structure; see Kaiser, E.T. and Kezdy, F.J. (1984) Science 223:249-255. The present invention includes mutants of the amino-acid sequences depicted herein which have an unaltered protein secondary structure or, if the structure is altered, the mutant has the biological activity retained to some degree.

CHART A

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                5                  10                  15                  20
Met Glu Asn Asn Ile Gln Asn Gln Cys Val Pro Tyr Asn Cys Leu Asn Asn Pro Glu Val
ATG GAG AAT AAT ATT CAA AAT CAA TGC GTA CCT TAC AAT TGT TTA AAT AAT CCT GAA GTA

               25                  30                  35                  40
Glu Ile Leu Asn Glu Glu Arg Ser Thr Gly Arg Leu Pro Leu Asp Ile Ser Leu Ser Leu
GAA ATA TTA AAT GAA GAA AGA AGT ACT GGC AGA TTA CCG TTA GAT ATA TCC TTA TCG CTT

               45                  50                  55                  60
Thr Arg Phe Leu Leu Ser Glu Phe Val Pro Gly Val Gly Val Ala Phe Gly Leu Phe Asp
ACA CGT TTC CTT TTG AGT GAA TTT GTT CCA GGT GTG GGA GTT GCG TTT GGA TTA TTT GAT

               65                  70                  75                  80
Leu Ile Trp Gly Phe Ile Thr Pro Ser Asp Trp Ser Leu Phe Leu Leu Gln Ile Glu Gln
TTA ATA TGG GGT TTT ATA ACT CCT TCT GAT TGG AGC TTA TTT CTT TTA CAG ATT GAA CAA

               85                  90                  95                  100
Leu Ile Glu Gln Arg Ile Glu Thr Leu Glu Arg Asn Arg Ala Ile Thr Thr Leu Arg Gly
TTG ATT GAG CAA AGA ATA GAA ACA TTG GAA AGG AAC CGG GCA ATT ACT ACA TTA CGA GGG

              105                 110                 115                 120
Leu Ala Asp Ser Tyr Glu Ile Tyr Ile Glu Ala Leu Arg Glu Trp Glu Ala Asn Pro Asn
TTA GCA GAT AGC TAT GAA ATT TAT ATT GAA GCA CTA AGA GAG TGG GAA GCA AAT CCT AAT

              125                 130                 135                 140
Asn Ala Gln Leu Arg Glu Asp Val Arg Ile Arg Phe Ala Asn Thr Asp Asp Ala Leu Ile
AAT GCA CAA TTA AGG GAA GAT GTG CGT ATT CGA TTT GCT AAT ACA GAC GAC GCT TTA ATA

              145                 150                 155                 160
Thr Ala Ile Asn Asn Phe Thr Leu Thr Ser Phe Glu Ile Pro Leu Leu Ser Val Tyr Val
ACA GCA ATA AAT AAT TTT ACA CTT ACA AGT TTT GAA ATC CCT CTT TTA TCG GTC TAT GTT

              165                 170                 175                 180
Gln Ala Ala Asn Leu His Leu Ser Leu Leu Arg Asp Ala Val Ser Phe Gly Gln Gly Trp
CAA GCG GCG AAT TTA CAT TTA TCA CTA TTA AGA GAC GCT GTA TCG TTT GGG CAG GGT TGG

              185                 190                 195                 200
Gly Leu Asp Ile Ala Thr Val Asn Asn His Tyr Asn Arg Leu Ile Asn Leu Ile His Arg
GGA CTG GAT ATA GCT ACT GTT AAT AAT CAT TAT AAT AGA TTA ATA AAT CTT ATT CAT AGA

              205                 210                 215                 220
Tyr Thr Lys His Cys Leu Asp Thr Tyr Asn Gln Gly Leu Glu Asn Leu Arg Gly Thr Asn
TAT ACG AAA CAT TGT TTG GAC ACA TAC AAT CAA GGA TTA GAA AAC TTA AGA GGT ACT AAT

              225                 230                 235                 240
Thr Arg Gln Trp Ala Arg Phe Asn Gln Phe Arg Arg Asp Leu Thr Leu Thr Val Leu Asp
ACT CGA CAA TGG GCA AGA TTC AAT CAG TTT AGG AGA GAT TTA ACA CTT ACT GTA TTA GAT

              245                 250                 255                 260
Ile Val Ala Leu Phe Pro Asn Tyr Asp Val Arg Thr Tyr Pro Ile Gln Thr Ser Ser Gln
ATC GTT GCT CTT TTT CCG AAC TAC GAT GTT AGA ACA TAT CCA ATT CAA ACG TCA TCC CAA

              265                 270                 275                 280
Leu Thr Arg Glu Ile Tyr Thr Ser Ser Val Ile Glu Asp Ser Pro Val Ser Ala Asn Ile
TTA ACA AGG GAA ATT TAT ACA AGT TCA GTA ATT GAG GAT TCT CCA GTT TCT GCT AAT ATA
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                285                 290                 295                 300
Pro Asn Gly Phe Asn Arg Ala Glu Phe Gly Val Arg Pro Pro His Leu Met Asp Phe Met
CCT AAT GGT TTT AAT AGG GCG GAA TTT GGA GTT AGA CCG CCC CAT CTT ATG GAC TTT ATG

                305                 310                 315                 320
Asn Ser Leu Phe Val Thr Ala Glu Thr Val Arg Ser Gln Thr Val Trp Gly Gly His Leu
AAT TCT TTG TTT GTA ACT GCA GAG ACT GTT AGA AGT CAA ACT GTG TGG GGA GGA CAC TTA

                325                 330                 335                 340
Val Ser Ser Arg Asn Thr Ala Gly Asn Arg Ile Asn Phe Pro Ser Tyr Gly Val Phe Asn
GTT AGT TCA CGA AAT ACG GCT GGT AAC CGT ATA AAT TTC CCT AGT TAC GGG GTC TTC AAT

                345                 350                 355                 360
Pro Gly Gly Ala Ile Trp Ile Ala Asp Glu Asp Pro Arg Pro Phe Tyr Arg Thr Leu Ser
CCT GGT GGC GCC ATT TGG ATT GCA GAT GAG GAT CCA CGT CCT TTT TAT CGG ACA TTA TCA

                365                 370                 375                 380
Asp Pro Val Phe Val Arg Gly Gly Phe Gly Asn Pro His Tyr Val Leu Gly Leu Arg Gly
GAT CCT GTT TTT GTC CGA GGA GGA TTT GGG AAT CCT CAT TAT GTA CTG GGG CTT AGG GGA

                385                 390                 395                 400
Val Ala Phe Gln Gln Thr Gly Thr Asn His Thr Arg Thr Phe Arg Asn Ser Gly Thr Ile
GTA GCA TTT CAA CAA ACT GGT ACG AAC CAC ACC CGA ACA TTT AGA AAT AGT GGG ACC ATA

                405                 410                 415                 420
Asp Ser Leu Asp Glu Ile Pro Pro Gln Asp Asn Ser Gly Ala Pro Trp Asn Asp Tyr Ser
GAT TCT CTA GAT GAA ATC CCA CCT CAG GAT AAT AGT GGG GCA CCT TGG AAT GAT TAT AGT

                425                 430                 435                 440
His Val Leu Asn His Val Thr Phe Val Arg Trp Pro Gly Glu Ile Ser Gly Ser Asp Ser
CAT GTA TTA AAT CAT GTT ACA TTT GTA CGA TGG CCA GGT GAG ATT TCA GGA AGT GAT TCA

                445                 450                 455                 460
Trp Arg Ala Pro Met Phe Ser Trp Thr His Arg Ser Ala Thr Pro Thr Asn Thr Ile Asp
TGG AGA GCT CCA ATG TTT TCT TGG ACG CAC CGT AGT GCA ACC CCT ACA AAT ACA ATT GAT

                465                 470                 475                 480
Pro Glu Arg Ile Thr Gln Ile Pro Leu Val Lys Ala His Thr Leu Gln Ser Gly Thr Thr
CCG GAG AGG ATT ACT CAA ATA CCA TTG GTA AAA GCA CAT ACA CTT CAG TCA GGT ACT ACT

                485                 490                 495                 500
Val Val Arg Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Arg Arg Thr Ser Gly Gly Pro
GTT GTA AGA GGG CCC GGG TTT ACG GGA GGA GAT ATT CTT CGA CGA ACA AGT GGA GGA CCA

                505                 510                 515                 520
Phe Ala Tyr Thr Ile Val Asn Ile Asn Gly Gln Leu Pro Gln Arg Tyr Arg Ala Arg Ile
TTT GCT TAT ACT ATT GTT AAT ATA AAT GGG CAA TTA CCC CAA AGG TAT CGT GCA AGA ATA

                525                 530                 535                 540
Arg Tyr Ala Ser Thr Thr Asn Leu Arg Ile Tyr Val Thr Val Ala Gly Glu Arg Ile Phe
CGC TAT GCC TCT ACT ACA AAT CTA AGA ATT TAC GTA ACG GTT GCA GGT GAA CGG ATT TTT

                545                 550                 555                 560
Ala Gly Gln Phe Asn Lys Thr Met Asp Thr Gly Asp Pro Leu Thr Phe Gln Ser Phe Ser
GCT GGT CAA TTT AAC AAA ACA ATG GAT ACC GGT GAC CCA TTA ACA TTC CAA TCT TTT AGT

                565                 570                 575                 580
Tyr Ala Thr Ile Asn Thr Ala Phe Thr Phe Pro Met Ser Gln Ser Ser Phe Thr Val Gly
TAC GCA ACT ATT AAT ACA GCT TTT ACA TTC CCA ATG AGC CAG AGT AGT TTC ACA GTA GGT
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                585                 590                 595                 600
Ala Asp Thr Phe Ser Ser Gly Asn Glu Val Tyr Ile Asp Arg Phe Glu Leu Ile Pro Val
GCT GAT ACT TTT AGT TCA GGG AAT GAA GTT TAT ATA GAC AGA TTT GAA TTG ATT CCA GTT

                605                 610                 615                 620
Thr Ala Thr Phe Glu Ala Glu Tyr Asp Leu Glu Arg Ala Gln Lys Ala Val Asn Ala Leu
ACT GCA ACA TTT GAA GCA GAA TAT GAT TTA GAA AGA GCA CAA AAG GCG GTG AAT GCG CTG

                625                 630                 635                 640
Phe Thr Ser Ile Asn Gln Ile Gly Ile Lys Thr Asp Val Thr Asp Tyr His Ile Asp Gln
TTT ACT TCT ATA AAC CAA ATA GGG ATA AAA ACA GAT GTG ACG GAT TAT CAT ATT GAT CAA

                645                 650                 655                 660
Val Ser Asn Leu Val Asp Cys Leu Ser Asp Glu Phe Cys Leu Asp Glu Lys Arg Glu Leu
GTA TCC AAT TTA GTG GAT TGT TTA TCA GAT GAA TTT TGT CTG GAT GAA AAG CGA GAA TTG

                665                 670                 675                 680
Ser Glu Lys Val Lys His Ala Lys Arg Leu Ser Asp Glu Arg Asn Leu Leu Gln Asp Pro
TCC GAG AAA GTC AAA CAT GCG AAG CGA CTC AGT GAT GAG CGG AAT TTA CTT CAA GAT CCA

                685                 690                 695                 700
Asn Phe Lys Gly Ile Asn Arg Gln Leu Asp Arg Gly Trp Arg Gly Ser Thr Asp Ile Thr
AAC TTC AAA GGC ATC AAT AGG CAA CTA GAC CGT GGT TGG AGA GGA AGT ACG GAT ATT ACC

                705                 710                 715                 720
Ile Gln Arg Gly Asp Asp Val Phe Lys Glu Asn Tyr Val Thr Leu Pro Gly Thr Phe Asp
ATC CAA AGA GGA GAT GAC GTA TTC AAA GAA AAT TAT GTC ACA CTA CCA GGT ACC TTT GAT

                725                 730                 735                 740
Glu Cys Tyr Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu Ser Lys Leu Lys Pro Tyr Thr
GAG TGC TAT CCA ACG TAT TTA TAT CAA AAA ATA GAT GAG TCG AAA TTA AAA CCC TAT ACT

                745                 750                 755                 760
Arg Tyr Gln Leu Arg Gly Tyr Ile Glu Asp Ser Gln Asp Leu Glu Ile Tyr Leu Ile Arg
CGT TAT CAA TTA AGA GGG TAT ATC GAG GAT AGT CAA GAC TTA GAA ATC TAT TTG ATC CGC

                765                 770                 775                 780
Tyr Asn Ala Lys His Glu Thr Val Asn Val Leu Gly Thr Gly Ser Leu Trp Pro Leu Ser
TAT AAT GCA AAA CAC GAA ACA GTA AAT GTG CTA GGT ACG GGT TCT TTA TGG CCG CTT TCA

                785                 790                 795                 800
Val Gln Ser Pro Ile Arg Lys Cys Gly Glu Pro Asn Arg Cys Ala Pro His Leu Glu Trp
GTC CAA AGT CCA ATC AGA AAG TGT GGA GAA CCG AAT CGA TGC GCG CCA CAC CTT GAA TGG

                805                 810                 815                 820
Asn Pro Asp Leu Asp Cys Ser Cys Arg Asp Gly Glu Lys Cys Ala His His Ser His His
AAT CCT GAT CTA GAT TGT TCC TGC AGA GAC GGG GAA AAA TGT GCA CAT CAT TCG CAT CAT

                825                 830                 835                 840
Phe Ser Leu Asp Ile Asp Val Gly Cys Thr Asp Leu Asn Glu Asp Leu Asp Val Trp Val
TTC TCC TTG GAC ATT GAT GTT GGA TGT ACA GAC TTA AAT GAG GAC TTA GAT GTA TGG GTG

                845                 850                 855                 860
Ile Phe Lys Ile Lys Thr Gln Asp Gly His Ala Arg Leu Gly Asn Leu Glu Phe Leu Glu
ATA TTC AAG ATT AAG ACG CAA GAT GGC CAT GCA AGA CTA GGA AAT CTA GAG TTT CTC GAA

                865                 870                 875                 880
Glu Lys Pro Leu Val Gly Glu Ala Leu Ala Arg Val Lys Arg Ala Glu Lys Lys Trp Arg
GAG AAA CCA TTA GTC GGG GAA GCA CTA GCT CGT GTG AAA AGA GCA GAG AAA AAA TGG AGA
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                885                 890                 895                 900
Asp Lys Arg Glu Lys Leu Glu Leu Glu Thr Asn Ile Val Tyr Lys Glu Ala Lys Glu Ser
GAT AAA CGT GAA AAA TTG GAA TTG GAA ACA AAT ATT GTT TAT AAA GAG GCA AAA GAA TCT

                905                 910                 915                 920
Val Asp Ala Leu Phe Val Asn Ser Gln Tyr Asp Gln Leu Gln Ala Asp Thr Asn Ile Ala
GTA GAT GCT TTA TTT GTA AAC TCT CAA TAT GAT CAA TTA CAA GCG GAT ACG AAT ATT GCC

                925                 930                 935                 940
Met Ile His Ala Ala Asp Lys Arg Val His Arg Ile Arg Glu Ala Tyr Leu Pro Glu Leu
ATG ATT CAT GCG GCA GAT AAA CGT GTT CAT AGA ATT CGG GAA GCG TAT CTT CCA GAG TTA

                945                 950                 955                 960
Ser Val Ile Pro Gly Val Asn Val Asp Ile Phe Glu Glu Leu Lys Gly Arg Ile Phe Thr
TCT GTG ATT CCG GGT GTA AAT GTA GAC ATT TTC GAA GAA TTA AAA GGG CGT ATT TTC ACT

                965                 970                 975                 980
Ala Phe Phe Leu Tyr Asp Ala Arg Asn Val Ile Lys Asn Gly Asp Phe Asn Asn Gly Leu
GCA TTC TTC CTA TAT GAT GCG AGA AAT GTC ATT AAA AAC GGT GAT TTC AAT AAT GGC TTA

                985                 990                 995                1000
Ser Cys Trp Asn Val Lys Gly His Val Asp Val Glu Glu Gln Asn Asn His Arg Ser Val
TCA TGC TGG AAC GTG AAA GGG CAT GTA GAT GTA GAA GAA CAA AAC AAC CAC CGT TCG GTC

               1005                1010                1015                1020
Leu Val Val Pro Glu Trp Glu Ala Glu Val Ser Gln Glu Val Arg Val Cys Pro Gly Arg
CTT GTT GTT CCG GAA TGG GAA GCA GAA GTG TCA CAA GAA GTT CGT GTC TGT CCG GGT CGT

               1025                1030                1035                1040
Gly Tyr Ile Leu Arg Val Thr Ala Tyr Lys Glu Gly Tyr Gly Glu Gly Cys Val Thr Ile
GGC TAT ATC CTT CGT GTC ACA GCG TAC AAG GAG GGA TAT GGA GAA GGT TGC GTA ACC ATT

               1045                1050                1055                1060
His Glu Ile Glu Asn Asn Thr Asp Glu Leu Lys Phe Ser Asn Cys Val Glu Glu Glu Val
CAT GAG ATC GAG AAC AAT ACA GAC GAA CTG AAG TTT AGC AAC TGC GTA GAA GAG GAA GTC

               1065                1070                1075                1080
Tyr Pro Asn Asn Thr Val Thr Cys Asn Asp Tyr Thr Ala Asn Gln Glu Glu Tyr Gly Gly
TAT CCA AAC AAC ACG GTA ACG TGT AAT GAT TAT ACT GCA AAT CAA GAA GAA TAC GGG GGT

               1085                1090                1095                1100
Ala Tyr Thr Ser Arg Asn Arg Gly Tyr Asp Glu Thr Tyr Gly Ser Asn Ser Ser Val Pro
GCG TAC ACT TCC CGT AAT CGT GGA TAT GAC GAA ACT TAT GGA AGC AAT TCT TCT GTA CCA

               1105                1110                1115                1120
Ala Asp Tyr Ala Ser Val Tyr Glu Glu Lys Ser Tyr Thr Asp Gly Arg Arg Asp Asn Pro
GCT GAT TAT GCG TCA GTC TAT GAA GAA AAA TCG TAT ACA GAT GGA CGA AGA GAC AAT CCT

               1125                1130                1135                1140
Cys Glu Ser Asn Arg Gly Tyr Gly Asp Tyr Thr Pro Leu Pro Ala Gly Tyr Val Thr Lys
TGT GAA TCT AAC AGA GGA TAT GGG GAT TAC ACA CCA CTA CCA GCT GGC TAT GTG ACA AAA

               1145                1150                1155                1160
Glu Leu Glu Tyr Phe Pro Glu Thr Asp Lys Val Trp Ile Glu Ile Gly Glu Thr Glu Gly
GAA TTA GAG TAC TTC CCA GAA ACC GAT AAG GTA TGG ATT GAG ATC GGA GAA ACG GAA GGA

               1165                1170
Thr Phe Ile Val Asp Ser Val Glu Leu Leu Leu Met Glu Glu
ACA TTC ATC GTG GAC AGC GTG GAA TTA CTC CTT ATG GAG GAA
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Claims

1. Bacillus thuringiensis PS81RRI, as available under Accession Number NRRLB-18484.

2. A toxin having the amino-acid sequence shown in Chart A, or a mutant thereof having activity against Spodoptera exigua, Plutella xylostella, and Choristoneura occidentalis, and is immunoreactive with antibodies to the sequence shown in Chart A; and wherein the sequence thereof is encoded by a DNA sequence which hybridises with the following sequence:

GGATACCGGTGACCCATTAACATTCCAATCTTTTAGTTACGC.

3. DNA encoding a Bacillus thuringiensis toxin according to claim 2.

4. DNA according to claim 3, having the nucleotide sequence shown in Chart A.

5. A recombinant DNA transfer vector comprising DNA according to claim 3 or claim 4.

6. A prokaryotic or eukaryotic host into which a DNA transfer vector according to claim 5 has been transferred and replicated.

7. A microorganism capable of expressing a Bacillus thuringiensis toxin having the amino-acid sequence shown in Chart A.

8. A microorganism according to claim 7, which is a species of Pseudomonas, Azotobacter, Erwinia, Serratia, Klebsiella, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter or Alcaligenes; a prokaryote selected from Enterbacteriaceae, Bacillaceae, Rhizobiaceae, Spirillaceae, Lactobacillaceae, Pseudomonadaceae, Azotobacteraceae and Nitrobacteraceae; or a lower eukaryote selected from Phycomycetes, Ascomycetes and Basidiomycetes.

9. A microorganism according to claim 8, which is Pseudomonas fluorescens or Escherichia coli.

10. A microorganism according to claim 9, which is E. coli (NM522) (pMYC 1603), as available under Accession Number NRRL B-18517.

11. A microorganism according to claim 7, which is a pigmented bacterium, yeast or fungus.

12. A microorganism according to any of claims 7 to 11, which is pigmented and phylloplane-adherent.

13. Substantially intact cells of a unicellular microorganism according to any of claims 1 and 6 to 12, containing the toxin.

14. Cells according to claim 13, as obtained by treatment with iodine or other chemical or physical means to prolong the insecticidal activity in the environment.

15. A composition comprising a microorganism according to any of claims 1 and 6 to 12, in association with an insecticide carrier or with formulation ingredients to be applied as a seed coating.

16. A composition according to claim 15, wherein the microorganism is in the form of spores or crystals.

17. A composition according to claim 15 or claim 16, wherein the carrier comprises beetle phagostimulants or attractants.

18. A method for controlling a lepidopteran insect pest, which comprises contacting the pest or its environment with a microorganism according to any of claims 1 and 6 to 12.

19. A method according to claim 18, wherein administration is to the rhizosphere, to the phylloplane, or to a body of water.

20. A method according to claim 18, which comprises placing a bait granule comprising the microorganism, e.g. as spores or crystals, on or in the soil when planting seed of a plant upon which the pest is known to feed.

21. A method according to claim 20, wherein the bait granule is placed at the same time as corn seed is planted in the soil.

22. Plasmid pMYC 1603, as available in a host according to claim 10.

Patentansprüche

1. Bacillus thuringiensis PS81RRI, wie erhältlich unter der Hinterlegungsnummer NRRL B-18484.

2. Toxin mit der in Tabelle A dargestellten Aminosäuresequenz, oder eine Mutante davon, das oder die Aktivität gegen Spodoptera exigua, Plutella xylostella und Choristoneura occidentalis aufweist und mit Antikörpern gegen die in Tabelle A dargestellte Sequenz immunreagiert, und wobei dessen bzw. deren Sequenz durch eine DNA-Sequenz kodiert wird, welche mit der folgenden Sequenz hybridisiert:

GGATACCGGTGACCCATTAACATTCCAATCTTTTAGTTACGC

3. DNA, die für ein Bacillus thuringiensis-Toxin nach Anspruch 2 kodiert.

4. DNA nach Anspruch 3, mit der in Tabelle A dargestellten Nukleotidsequenz.

5. Rekombinanter DNA-Transfer-Vektor, der DNA nach Anspruch 3 oder Anspruch 4 umfaßt.

6. Prokaryotischer oder eukaryotischer Wirt, in den ein DNA-Transfer-Vektor nach Anspruch 5 transferiert und repliziert worden ist.

7. Mikroorganismus, der imstande ist, ein Bacillus thuringiensis-Toxin mit der in Tabelle A dargestellten Aminosäuresequenz zu exprimieren.

8. Mikroorganismus nach Anspruch 7, der eine Spezies ist von Pseudomonas, Azotobacter, Erwinia, Serratia, Klebsiella, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter oder Alcaligenes; ein aus Enterobacteriaceae, Bacillaceae, Rhizobiaceae, Spirillaceae, Lactobacillaceae, Pseudomonadaceae, Azotobacteraceae und Nitrobacteraceae ausgewählter Prokaryot; oder ein aus Phycomycetes, Ascomycetes und Basidiomycetes ausgewählter niederer Eukaryot.

9. Mikroorganismus nach Anspruch 8, der Pseudomonas fluorescens oder Escherichia coli ist.

10. Mikroorganismus nach Anspruch 9, der E. coli (NM522) (pMYC 1603) ist, wie erhältlich unter der Hinterlegungsnummer NRRL B-18517.

11. Mikroorganismus nach Anspruch 7, der ein pigmentiertes Bakterium, Hefe oder ein Pilz ist.

12. Mikroorganismus nach irgendeinem der Ansprüche 7 bis 11, der pigmentiert ist und Phyllooberflächen-adhärent.

13. Im wesentlichen intakte Zellen eines einzelligen Mikroorganismus nach irgendeinem der Ansprüche 1 und 6 bis 12, der das Toxin enthält.

14. Zellen nach Anspruch 13, wie erhalten durch Behandlung mit Iod oder anderen chemischen oder physikalischen Mitteln, um die insektizide Aktivität in der Umwelt zu verlängern.

15. Zusammensetzung, umfassend einen Mikroorganismus nach irgendeinem der Ansprüche 1 und 6 bis 12 in Verbindung mit einem Insektizid-Träger oder mit Formulierungsbestandteilen für die Anwendung als Samenüberzug.

16. Zusammensetzung nach Anspruch 15, worin der Mikroorganismus in Form von Sporen oder Kristallen vorliegt.

17. Zusammensetzung nach Anspruch 15 oder Anspruch 16, worin der Träger Phagostimulantien oder Lockstoffe für Insekten umfaßt.

18. Verfahren zur Kontrolle eines Lepidoptera-Insekten-Schädlings, welches umfaßt, daß der Schädling oder dessen

Umwelt mit einem Mikroorganismus nach irgendeinem der Ansprüche 1 und 6 bis 12 in Kontakt gebracht wird.

19. Verfahren nach Anspruch 18, worin die Ausbringung in der Rhizosphäre, der Phyllooberfläche oder einem Wasserkörper erfolgt.

20. Verfahren nach Anspruch 18, welches umfaßt, daß ein Köderkörnchen, welches den Mikroorganismus, z.B. als Sporen oder Kristalle, umfaßt, auf oder in dem Erdreich ausgebracht wird, wenn Samen einer Pflanze, von der sich der Schädling bekanntermaßen ernährt, gepflanzt wird.

21. Verfahren nach Anspruch 20, worin das Köderkörnchen gleichzeitig mit dem Einpflanzen von Getreidesamen in das Erdreich ausgebracht wird.

22. Plasmid pMYC 1603, wie erhältlich in einem Wirt nach Anspruch 10.

Revendications

1. Bacillus thuringiensis PS81RRI, disponible sous le numéro d'accession NRRLB-18484.

2. Toxine ayant la séquence d'acides aminés présentée dans le diagramme A, ou mutant de celle-ci ayant une activité contre Spodoptera exigua, Plutella xylostella et Choristoneura occidentalis, et qui est immunoréactive avec des anticorps contre la séquence présentée dans le diagramme A, cette séquence étant codée par une séquence d'ADN qui s'hybride avec la séquence suivante :

GGATACCGGTGACCCATTAACATTCCAATCTTTTAGTTACGC.

3. ADN codant pour une toxine de Bacillus thuringiensis selon la revendication 2.

4. ADN selon la revendication 3, ayant la séquence nucléotidique présentée dans le diagramme A.

5. Vecteur de transfert d'ADN recombinant comprenant de l'ADN conforme à la revendication 3 ou à la revendication 4.

6. Hôte procaryote ou eucaryote dans lequel un vecteur de transfert d'ADN conforme à la revendication 5 a été transféré et répliqué.

7. Micro-organisme capable d'exprimer une toxine de Bacillus thuringiensis ayant la séquence d'acides aminés présentée dans le diagramme A.

8. Micro-organisme selon la revendication 7, qui est une espèce de Pseudomonas, d'Azotobacter, d'Erwinia, de Serratia, de Klebsiella, de Rhizobium, de Rhodopseudomonas, de Méthylophilius, d'Agrobacterium, d'Acetobacter ou d'Alcaligenes ; procaryote choisi parmi les entérobactériacées, les bacillacées, les rhizobiacées, les spirillacées, les lactobacillacées, les pseudomonadacées, les azotobactéracées et les nitrobactéracées ; ou eucaryote inférieur choisi parmi les phycomycètes, les ascomycètes et les basidiomycètes.

9. Micro-organisme selon la revendication 8, qui est Pseudomonas fluorescens ou Escherichia coli.

10. Micro-organisme selon la revendication 9, qui est E. coli (NM522) (pMYC 1603), disponible sous le numéro d'accession NRRL B-18517.

11. Micro-organisme selon la revendication 7, qui est une bactérie, une levure ou un champignon pigmenté.

12. Micro-organisme selon l'une quelconque des revendications 7 à 11, qui est pigmenté et adhére au phylloplan.

13. Cellule pratiquement intacte d'un micro-organisme unicellulaire selon l'une quelconque des revendications 1 et 6 à 12, contenant la toxine.

14. Cellules selon la revendication 13, obtenues par traitement avec de l'iode ou un autre moyen chimique ou physique permettant de prolonger l'activité insecticide dans l'environnement.

15. Composition comprenant un micro-organisme selon l'une quelconque des revendications 1 et 6 à 12, en association avec un véhicule pour insecticide ou avec des ingrédients de formulation, à appliquer sous forme de revêtement de semence.

16. Composition selon la revendication 15, dans laquelle le micro-organisme est sous forme de spores ou de cristaux.

17. Composition selon la revendication 15 ou la revendication 16, dans laquelle le véhicule comprend des phagostimulants ou des attractifs pour coléoptères.

18. Procédé de lutte contre un lépidoptère nuisible, qui comprend la mise en contact du nuisible ou de son milieu avec un micro-organisme selon l'une quelconque des revendications 1 et 6 à 12.

19. Procédé selon la revendication 18, dans lequel l'administration se fait sur la rhizosphère, sur le phylloplan ou sur un volume d'eau.

20. Procédé selon la revendication 18, qui comprend le fait de placer un granule appât contenant le micro-organisme, par exemple sous forme de spores ou de cristaux, sur ou dans le sol au moment du semis de la graine d'une plante sur laquelle on sait que le nuisible se nourrit.

21. Procédé selon la revendication 20, dans lequel le granule appât est placé au moment même où le grain de céréale est semé dans le sol.

22. Plasmide pMYC 1603, disponible dans un hôte selon la revendication 10.

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